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(54) Title: VECTOR VACCINES OF RECOMBINANT FELINE HERPESVIRUS (57) Abstract The present invention is concerned with a Feline herpesvirus (FHV) mutant comprising a heterologous gene introduced into a section of the FHV genome. The invention also relates to a vector vaccine comprising such an FHV mutant which expresses a heterologous polypeptide derived from a feline pathogen and induces an adequate immune response in an inoculated host against both FHV and the feline pathogen.		

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Vector vaccines of recombinant Feline herpesvirus

The present invention is concerned with a feline herpesvirus (FHV) mutant comprising a mutation in a section of the FHV genome, a nucleic acid sequence comprising said section of the FHV genome, a nucleic acid sequence comprising a heterologous DNA sequence flanked by DNA derived from said section, a recombinant DNA molecule comprising such nucleic acid sequences, a cell culture infected with an FHV mutant, as well as vaccine comprising the FHV mutant.

One of the major clinical problems in diseases of Felidae is associated with respiratory tract infections. The great majority of these cases are caused by either feline herpesvirus 1 (FHV) or feline calicivirus.

FHV is the causative agent of feline viral rhinotracheitis in cats. In kittens, FHV infection can generalize resulting in mortality rates of up to 50%. The disease is common and is found world-wide and is characterized by sneezing, depression, and ocular and nasal discharge.

The FHV is a member of the family Herpes-viridae, subfamily α -herpesvirus. The genome is about 126 kb in length and is composed of a unique long (U_L) region of about 99 kb and a short region of 27 kb comprising an unique short (U_S) region of about 9 kb flanked by inverted repeats of about 8 kb (Grail et al., Arch. Virol. 116, 209-220, 1991).

Because of the prevalence and seriousness of FHV infection, feline viral rhinotracheitis vaccines comprising modified live or killed FHV have been developed and have resulted in a successful reduction of the incidence of the disease.

In addition to FHV infection, cats are also susceptible to infection by various other pathogens, such as feline leukemia virus, feline calicivirus,

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feline immunodeficiency virus, feline coronavirus and feline Chlamydia.

At present, in general, cats can be protected against infection by these pathogenic micro-organisms with live or inactivated vaccines.

However, these types of vaccines may suffer from a number of drawbacks. Using attenuated live vaccines always involves the risk of inoculating animals with inadequately attenuated pathogenic micro-organisms. In addition, the attenuated pathogens may revert to a virulent state resulting in disease of the inoculated animals and the possible spread of the pathogen to other animals.

Inactivated vaccines generally induce only a low level of immunity, requiring repeated immunizations. Furthermore, the neutralization inducing antigenic determinants of the pathogens may become altered by the inactivation treatment, decreasing the protective potency of the vaccine.

Moreover, a problem with combined live viral vaccines is the mutual influence of the antigenic components resulting in a decrease of the potency of one or more of the constituting components.

Furthermore, with currently administered live attenuated or inactivated FHV vaccines it is not possible to determine whether a specific animal is a carrier of an FHV field virus or whether the animal was vaccinated. Hence, it is important to discriminate between animals vaccinated with an FHV vaccine and those infected with a field virus so as to be able to take appropriate measures to reduce spreading of a virulent field virus. The introduction of for example a serologically identifiable marker can be achieved by introducing a mutation in genes encoding non-essential (glyco) proteins of the FHV which normally give rise to the production of antibodies in an infected host animal.

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It is an object of the present invention to provide an FHV mutant which can be used not only for the preparation of a vaccine against feline viral rhinotracheitis but also against other infectious diseases of Felidae, which obviates any potential risk associated with the use of a live attenuated pathogen as a vaccine, which stimulates both the humoral and cellular immune system in a potent way without the explicit need of an adjuvant and which offers the possibility of a multivalent vaccine without the risk of adverse mutual interference of different antigenic components.

An other object of the present invention is to provide an FHV vaccine virus which is distinguishable from any field strain or any other FHV vaccine virus.

The present invention provides an FHV mutant comprising a mutation in a section of an FHV genome which spans from the upstream non-coding region of open reading frame-1 up to and including the downstream non-coding region of open reading frame-6 localized within a DNA fragment of the FHV genome having a restriction enzyme map essentially defined by figure 1.

A mutation is understood to be a change of the genetic information in the above-mentioned section with respect to the genetic information present in this section of the genome of the parent FHV.

The mutation is in particular, a nucleic acid substitution, deletion, insertion or inversion, or a combination thereof resulting in a FHV mutant which fails to produce one or more antigenic or functional polypeptides shown in SEQ ID NO: 2-7, or in an FHV mutant which contains an inserted heterologous nucleic acid sequence.

The FHV mutant according to the present invention can be derived from any FHV strain, e.g. strain G2620

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(commercially available from Intervet International B.V., the Netherlands), C-27 (ATCC VR-636), FVRm (ATCC VR-814), FVRm vaccine (ATCC VR-815) or F2.

The term "polypeptide" as used herein refers to a molecular chain of amino acids, does not refer to a specific length of the product and if required can be modified in vivo or in vitro, for example by glycosylation, amidation, carboxylation or phosphorylation; thus inter alia peptides, oligopeptides and proteins are included within the definition of polypeptide.

The prerequisite for a useful FHV mutant according to the present invention is that the mutation is introduced in a permissive position or region of the genomic FHV sequence, i.e. a position or region which can be used for the incorporation of the mutation without disrupting essential functions of FHV such as those necessary for infection or replication.

Until now little is known about the localization of genes in the FHV genome. Rota et al. (Virology 154, 168-179, 1986) and Grail et al. (Arch. Virol. 116, 209-220, 1991) disclosed physical maps of the FHV genome.

Nünberg et al. (J. Virology 63, 3240-3249, 1989) and Cole et al. (J. Virology 64, 4930-4938, 1990) identified the thymidine kinase (TK) gene and mapped this gene in the SalI-A restriction fragment (Rota et al., supra) of the FHV genome. Subsequently, several recombinant FHV strains were constructed in which FeLV env and gag genes have been inserted within the FHV TK gene.

The section of the FHV genome referred to in the present invention has not been identified previously within the FHV genome. Surprisingly, it has been found that a mutation such as the incorporation of

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heterologous DNA is allowable in this region without disrupting essential functions of the FHV.

The section of the FHV genome used to introduce one or more mutations in order to prepare a FHV mutant according to the invention is located within a 13.5 kb restriction fragment generated by partial digestion of genomic FHV DNA with the enzyme Sau3A (figure 1).

Said fragment is analyzed in detail by restriction enzyme mapping and essentially corresponds to a region within the U_S segment of the viral genome between map unit 0.87 and 0.96 on the map of Grail et al. (supra).

The section of the FHV genome used in the present invention for the introduction of one or more mutations is located within two adjacent BamHI fragments of 1.9 and 5.2 kb, is about 6.1 kb in length and comprises the DNA sequence of six consecutive open reading frames (ORF) as well as the intergenic sequences flanking these open reading frames, including the upstream non-coding sequence of ORF-1 and the downstream non-coding sequence of ORF-6.

These flanking intergenic sequences do not form part of an ORF or protein encoding DNA sequence, or do not comprise sequences regulating the replication of the virus. Said flanking sequences extend in the upstream and downstream direction up to the start or end of the nearest open reading frame.

In particular, the present invention provides an FHV mutant containing a mutation in a section of the FHV genome which spans the region comprising the DNA sequence of ORF 1-6 encoding polypeptides shown in SEQ ID NO: 2-7 and intergenic flanking sequences thereof, and more preferably the region comprising the DNA sequence shown in SEQ ID NO: 1.

ORF-1 is an open reading frame located between nucleotide positions 127 and 1281 (SEQ ID NO: 1) and encodes a polypeptide of 384 amino acids

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(SEQ ID NO: 2) and contains the unique BglIII site at nucleotide position 1210 used for the insertion of the β -galactosidase marker gene, thereby indicating that the hypothetical polypeptide encoded by ORF-1 does not have essential functions for the infection of cells or replication of the virus.

ORF-2 starts at nucleotide position 1460 and continues up to nucleotide position 3058 (SEQ ID NO: 1) encoding a polypeptide of 532 amino acids (SEQ ID NO: 3). The β -galactosidase marker gene was also inserted into one of the EcoRV sites of this ORF.

ORF-3 is a small open reading frame and is identified in an other phase than ORF-2 and shares a common nucleotide-sequence with ORF-2. ORF-3 is located between nucleotide position 3055 and 3357 (SEQ ID NO: 1) and encodes a polypeptide of 100 amino acids (SEQ ID NO: 4). The Spe I site was selected for the insertion of the β -galactosidase marker gene.

ORF-4 starts at nucleotide position 3505 and continues up to nucleotide position 3963 (SEQ ID NO: 1) encoding a polypeptide of 152 amino acids (SEQ ID NO: 5).

Both ORF-5 and ORF-6 are translated in the reverse orientation towards the internal repeated sequence. ORF-5 is located between nucleotide positions 4256 and 4897 encoding a polypeptide of 213 amino acids (SEQ ID NO: 1 complementary, SEQ ID NO: 6). ORF-6 is located between nucleotide positions 5138 and 6142 encoding a polypeptide of 334 amino acids (SEQ ID NO: 1 complementary, SEQ ID NO: 7).

The Sau3A site at position 5737 (SEQ ID NO: 1, ORF-6) has also been used as an insertion site for the incorporation of the heterologous β -galactosidase marker gene resulting in a viable virus indicating that this region is not essential for viral infectivity and replication.

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Particularly, the mutation introduced into an FHV in order to obtain an FHV mutant according to the present invention is introduced within one or more open reading frames as defined above, preferably in ORF-1, ORF-2, ORF-3 and/or ORF-6.

Surprisingly, it has further been found that the introduction of a mutation into ORF-1 significantly reduces the virulence of the live FHV mutant without affecting the protective properties of the FHV mutant significantly. This finding has offered the possibility to obtain an attenuated FHV mutant, e.g. by introducing a deletion or insertion into the region defined above, which mutant can be administered safely to the animals to be vaccinated in a live form, even via the oro-nasal route.

It will be understood that for the DNA sequence of the FHV genome, natural variations can exist between individual FHV viruses. These variations may result in deletions, substitutions, insertions, inversions or additions of one or more nucleotides. These FHV variants may encode corresponding ORFs that differ from the ORFs disclosed herein. The DNA sequence of such variant ORFs can be located by several methods, including hybridization with the DNA sequence provided in SEQ ID NO: 1, or comparison of the physical map to locate analogous regions comprising said ORFs. Therefore, the present invention provides a section of the FHV genome which allows the introduction of a mutation as defined above, obtainable from any strain of FHV.

Moreover, the potential exists to use genetic engineering technology to bring about above-mentioned variations resulting in a DNA sequence related to the DNA sequence of the section defined above. It is clear that an FHV mutant comprising a mutation incorporated into said section of the FHV genome characterized by

such a related DNA sequence is also included within the scope of the present invention.

In a preferred embodiment of the present invention an FHV mutant is provided wherein the mutation comprises the insertion of a heterologous DNA sequence in the FHV genome.

The heterologous DNA sequence to be incorporated into the FHV genome is a DNA sequence which either does encode a polypeptide different from the polypeptide encoded by the target ORF or is a non-coding DNA sequence, and can be derived from any source, e.g. viral, eukaryotic, prokaryotic or synthetic, including oligonucleotides suitable for the interruption of the expression of the gene products from the ORFs disclosed above.

Such a suitable oligonucleotide may comprise three translational stop codons in each of the possible reading frames in both directions, in addition to one or more appropriate restriction enzyme cleavage sites useful for the insertion of a second heterologous DNA sequence.

In particular, the heterologous DNA sequence encodes an antigen of a significant pathogen for feline species which is able to elicit a protective immune response, said antigen being expressed by the FHV mutant according to the invention upon replication in a host cell.

Preferably DNA sequences encoding an antigen of feline leukemia virus, feline immuno-deficiency virus, feline calicivirus, feline parvo-virus, feline coronavirus and feline Chlamydia are contemplated for incorporation into the section of the FHV genome disclosed herein.

Furthermore, nucleic acid sequences encoding polypeptides for pharmaceutical or diagnostic applications, in particular immuno-modulators such as

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lymphokines, interferons or cytokines, may be incorporated into said section.

Moreover, as the open reading frames disclosed herein do not display essential functions, one or more of these regions may be deleted partially or completely resulting in the interruption of the expression of an antigenic or functional gene product of the respective open reading frame, if desired followed by the incorporation of a heterologous DNA sequence into the deletion.

An essential requirement for the expression of the heterologous DNA sequence by the FHV mutant according to the invention is an adequate promotor operably linked to the heterologous DNA sequence. It is obvious to those skilled in the art that the choice of a promotor extends to any eukaryotic, prokaryotic or viral promotor capable of directing gene transcription in cells infected by the FHV mutant, e.g. promoters of the retroviral long terminal repeat (Gorman et al., Proc. Natl. Acad. Sci. USA 79, 6777-6781, 1982), the SV40 promotor (Mulligan and Berg, Science 209, 1422-1427, 1980) or the cytomegalovirus immediate early promotor (Schaffner et al., Cell 41, 521-530, 1985).

The technique of in vivo homologous recombination can be used to introduce the heterologous DNA sequence into the FHV genome. This is accomplished by first constructing a recombinant DNA molecule for recombination with FHV genomic DNA. Such a molecule may be derived from any suitable plasmid, cosmid or phage, plasmids being most preferred, and contains a heterologous DNA sequence, if desired operably linked to a promotor. Said DNA sequence and promotor are introduced into a fragment of genomic FHV DNA containing the whole or part of the non-essential

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section of the FHV genome as defined herein, subcloned in the recombinant DNA molecule.

These so called insertion-region sequences which flank the heterologous DNA sequence should be of appropriate length, e.g. 50-3000 bp, as to allow in vivo homologous recombination with the viral FHV genome to occur. If desired, a construct can be made which contains two or more different heterologous DNA sequences derived from the same or different pathogens said sequences being flanked by insertion-region sequences of FHV defined herein. Such a recombinant DNA molecule can be employed to produce recombinant FHV which expresses two or more different antigenic polypeptides to provide a multivalent vaccine.

Secondly, cells, e.g. feline kidney cells (CRFK) or feline embryo cells can be transfected with FHV DNA in the presence of the recombinant DNA molecule containing the heterologous DNA sequence flanked by appropriate FHV sequences whereby recombination occurs between the insertion-region sequences in the recombinant DNA molecule and the insertion-region sequences in the FHV genome. Recombination can also be brought about by transfecting the infected cells with a nucleic acid sequence containing the heterologous DNA sequence flanked by appropriate flanking insertion-region sequences. Recombinant viral progeny is thereafter produced in cell culture and can be selected for example genotypically or phenotypically, e.g. by hybridization, detecting enzyme activity encoded by a gene co-integrated along with the heterologous DNA sequence or detecting the antigenic heterologous polypeptide expressed by the recombinant FHV immunologically. Recombinant virus can also be selected positively based on resistance to compounds such as neomycine, gentamycine or mycophenolic acid. The selected recombinant FHV can be cultured on a large scale in cell culture whereafter recombinant FHV

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containing material or heterologous polypeptides expressed by said FHV can be collected therefrom.

In case a deletion mutant according to the invention is desired, either partial or complete deletion of the region from the viral genome identified above can be achieved by the technique of in vivo homologous recombination.

First, a DNA fragment comprising part of the unique short sequence as defined in SEQ ID No.:1 and flanked by at least 100 nucleotides on either site, can be subcloned into a convenient plasmid vehicle.

The deletion to be introduced in the described region can be made in this plasmid by a restriction digest with one or more enzymes of which the sites are correctly positioned in or near the open reading frame. Recircularization of the remaining plasmid molecule would result in a derivative lacking at least part of the coding sequence present within the newly identified region. Alternatively, progressive deletions can be introduced either in one or two directions starting from within a restriction site present within the sequence of an open reading frame. Enzymes such as *BalI* or endonuclease *III* can be used for this purpose. Recircularized plasmid molecules are transformed into *E.coli* cells and individual colonies are analyzed by restriction mapping in order to determine the size of the deletion introduced into the specified region. An accurate positioning of the deletion can be obtained by sequence analysis. The plasmid containing a defined deletion can be cotransfected with FHV viral DNA into cultured feline cells. After in vivo recombination has occurred, the deletion will be introduced at the correct position within the described region of the viral genome. Recombinants among the viral progeny can be identified for example by means of 15 to 20 bases long synthetic oligomer which hybridizes specifically to the

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nucleotide sequence which is generated at the junction where the deletion originally was introduced.

A. live FHV mutant according to the present invention, and in particular a live FHV expressing one or more different heterologous polypeptides of specific pathogens, can be used to vaccinate animals, particularly domestic and non-domestic cats or canine species. Vaccination with such a live vector vaccine is preferably followed by replication of the FHV mutant within the inoculated host, expressing in vivo the heterologous polypeptide along with the FHV polypeptides. The polypeptides expressed in the inoculated host will then elicit an immune response against both FHV and the specific pathogen. If the heterologous polypeptide derived from the specific pathogen can stimulate a protective immune response, then the animal inoculated with the FHV mutant according to the invention will be immune to subsequent infection by that pathogen as well as to infection by FHV. Thus, a heterologous nucleic acid sequence incorporated into the insertion-region of the FHV genome according to the invention may be continuously expressed in vivo, providing a solid, safe and longlasting immunity to a pathogen.

An FHV mutant according to the invention containing and expressing one or more different heterologous polypeptides can serve as a monovalent or multivalent vaccine.

For the preparation of a live vaccine the recombinant FHV mutant according to the present invention can be grown on a cell culture of feline origin. The viruses thus grown can be harvested by collecting the tissue cell culture fluids and/or cells. The live vaccine may be prepared in the form of a suspension or may be lyophilized.

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In addition to an immunogenically effective amount of the recombinant FHV the vaccine may contain a pharmaceutically acceptable carrier or diluent.

Examples of pharmaceutically acceptable carriers or diluents useful in the present invention include stabilizers such as SPGA, carbohydrates (e.g. sorbitol, mannitol, starch, sucrose, glucose, dextran), proteins such as albumin or casein, protein containing agents such as bovine serum or skimmed milk and buffers (e.g. phosphate buffer).

Optionally, one or more compounds having adjuvant activity may be added to the vaccine. Suitable adjuvants are for example aluminium hydroxide, phosphate or oxide, oil-emulsions (e.g. of Bayol F(R) or Marcol 52(R), saponins or vitamin-E solubilisate.

The useful dosage to be administered will vary depending on the age, weight, mode of administration and type of pathogen against which vaccination is sought. A suitable dosage can be for example about $10^{3.0}$ - $10^{7.0}$ pfu/animal.

An FHV mutant according to the invention can also be used to prepare an inactivated vaccine.

For administration to animals, the FHV mutant according to the present invention can be given *inter alia* intranasally, intradermally, subcutaneously or intramuscularly.

It is a further object of the present invention to produce subunit vaccines, pharmaceutical and diagnostic preparations comprising a heterologous polypeptide expressed by an FHV mutant according to the invention. This can be achieved by culturing cells infected with said FHV under conditions that promote expression of the heterologous polypeptide. The heterologous polypeptide may then be purified with conventional techniques to a certain extent depending on its intended use and processed further into a

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preparation with immunizing, therapeutic or diagnostic activity.

The above described active immunization against specific pathogens will be applied as a protective treatment in healthy animals. It goes without saying that animals already infected with a specific pathogen can be treated with antiserum comprising antibodies evoked by a FHV mutant according to the invention comprising a heterologous gene derived from the specific pathogen encoding an antigenic polypeptide. Antiserum directed against a recombinant FHV according to the invention can be prepared by immunizing animals, for example cats, with an effective amount of said FHV mutant in order to elicit an appropriate immune response. Thereafter the animals are bled and antiserum can be prepared.

A further object of the present invention is to provide a nucleic acid sequence encoding an FHV polypeptide which can be applied for the preparation of a vaccine for the immunization of feline species against FHV infection and for the preparation of a diagnostic test.

Such a nucleic acid sequence is characterized in that it contains at least part of ORF-1 or ORF-2 encoding a polypeptide having the amino acid sequence as shown in SEQ ID NO: 2 and 3, respectively.

Preferably, the nucleic acid sequence comprises at least part of the DNA sequence of ORF-1 or ORF-2 having the nucleotide sequence located between nucleotide position 127-1281 and 1460-3058 (SEQ ID NO:1), respectively.

A nucleic acid sequence according to the present invention can be ligated to various replication effecting DNA sequences with which it is not associated or linked in nature, optionally containing portions of

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DNA encoding fusion protein sequences such as β -galactosidase, resulting in a so called recombinant DNA molecule which can be used for the transformation of a suitable prokaryotic or eukaryotic host. Such hybrid DNA molecules, are preferably derived from, for example plasmids, or from nucleic acid sequences present in bacteriophages, cosmids or viruses.

In general, prokaryotes are preferred for cloning of DNA sequences in constructing the vectors useful in the invention. For example E.coli K12 is particularly useful. Other E.coli strains may be used include such as DH5A or JM101.

For expression nucleic acid sequences of the present invention are operably linked to expression control sequences. Such control sequences may comprise promoters, enhancers, operators and ribosome binding sites.

The present invention also comprises a polypeptide displaying immunological characteristics of the polypeptide encoded by ORF-1 or ORF-2 having an amino acid sequence as shown in ID NO: 2 and 3, respectively, i.e. the polypeptide comprises one or more immunoreactive and/or antigenic determinants of the polypeptide encoded by ORF-1 or ORF-2, essentially free from the whole virus or other proteins with which it is ordinarily associated.

Immunization of cats against FHV infection can, for example be achieved by administering to the animals a polypeptide according to the invention in an immunologically relevant context as a so-called subunit vaccine.

An alternative to subunit vaccines are live vector vaccines. A nucleic acid sequence according to the invention is introduced by recombinant DNA

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techniques into a micro-organism (e.g. a bacterium or virus) in such a way that the recombinant micro-organism is still able to replicate thereby expressing a polypeptide coded by the inserted nucleic acid sequence.

The FHV polypeptides encoded by ORF 1 or 2 as described above can be used to produce antibodies, both polyclonal, monospecific and monoclonal. Antibodies or antiserum directed against a polypeptide according to the invention have potential use in passive immunotherapy, diagnostic immunoassays and generation of anti-idiotypic antibodies.

The invention also relates to an "immunochemical reagent", which reagent comprises at least one of the polypeptides according to the invention or an antigenic fragment thereof.

The term "immunochemical reagent" signifies that the polypeptides according to the invention have been bound to a suitable support or have been provided with a labelling substance.

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EXAMPLE 1Characterization of a new insertion region in the unique short sequence of the FHV genome.

- Preparation of FHV DNA and establishment of a genomic library in lambda vector EMBL4.

The vaccine strain of FHV-1 (commercially available as feline rhinotracheitis virus, strain G2620, from Intervet International B.V.; Holland) was grown on Crandell-Rees feline kidney (CRFK) cells (Crandell, R.A. et al., In Vitro 9, 176-185, 1973) in Glasgow's modified minimum essential medium supplemented with 2.0 g/l tryptose, 2.5 g/l lactalbumin hydrolysate and 5% foetal calf serum. Culture super-natants were harvested after full cytopathic effect had developed and virus was concentrated by precipitation with polyethylene glycol (Yamamoto, K.R. et al., Virology 40, 734-744, 1970). DNA was released from virus particles by digestion at 37°C for two hours with 100 µg/ml proteinase K (Promega, Wisconsin, USA) in a buffer containing 20mM Tris-HCl (pH 7.5), 10 mM EDTA and 0.5% SDS. After repeated extractions with a 1:1 mixture of phenol/chloroform, nucleic acids were precipitated with two volumes of ethanol and dissolved in TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). Viral DNA was partially digested with the restriction enzyme Sau3A (Promega, Wisconsin, USA) according to the conditions recommended by the enzyme supplier and reaction products were separated on a preparative 0.8% agarose gel.

Fragments of the size fraction between 10 and 15 kb were isolated and ligated 2 hours at 15°C with DNA from bacteriophage lambda EMBL4 digested with BamHI

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and SalI (Kaiser, K. and Murray, N. in "DNA Cloning", Volume 1, Chapter 1, IRL Press, 1985). Reaction products were packaged in vitro (Promega, Wisconsin, USA) and recombinant phage was plated on E.coli host strain LE392. The library in lambda EMBL4 was enriched for recombinants containing inserts with sequences specifically present in relatively large SalI restriction fragments of the viral genome by screening nitrocellulose replica filters with a ³²P-labelled DNA probe consisting of 10-15 kb restriction fragments isolated by preparative agarose gel electrophoresis of FHV genomic DNA digested with SalI (for technical details see Sambrook, J. et al., in "Molecular Cloning: A laboratory manual", Chapter 2, Cold Spring Harbor Laboratory Press, 1989). Individual recombinants obtained from these screening procedures were amplified and the restriction pattern of the lambda insert DNA was compared with the published map of the complete FHV genome (Grail, A. et al., Arch. Virol., 116, 209-220, 1991). One of the isolates designated λFHV04, was selected for further study and the 13.5 kb insert of this clone (see figure 1) was positioned in the unique short segment of the viral genome between unit 0.87 and 0.96 on the map of Grail et al., supra.

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EXAMPLE 2Insertion of a marker gene at restriction sites in the unique short genome segment of FHV.

The 4.8 kb SacI fragment of λ FHV04 which was subcloned in pGEM3Z resulting in pFHV13 (see figure 2A), revealed a unique BglII restriction site in a suitable position for the integration of a marker gene. The gene used for insertion was derived from pCH110 (Pharmacia, Uppsala, Sweden) by replacing a 72 bp SphI fragment near the SV40 origin of replication by a 12-base double stranded synthetic oligonucleotide containing the recognition sequences for both BamHI and SalI, and single-stranded extremities compatible with the ends generated after digestion of DNA with SphI.

Insertion of the linker between the two SphI restriction sites of pCH110 does not restore the recognition sequence for SphI on either site and creates both a BamHI and SalI site upstream of the SV40 early promoter. Subsequent digestion with BamHI generated a 4.0 kb β -galactosidase expression cassette which was inserted at the BglII site of pFHV13 resulting in pFHV19 (see figure 2B). Linearized DNA of plasmid pFHV19 was introduced together with viral DNA into CRFK cells by calciumphosphate-mediated DNA precipitation (Graham, F. L. and v.d. Eb, A. J., Virology 52, 456-467, 1973). One microgram of DNA from pFHV19 were mixed with 15 microgram of DNA from FHV infected cells in a final volume of 376 μ l H₂O and added to 500 μ l of 2x HBSP (10 mM KCl, 280 mM NaCl, 12 mM glucose, 1.5 mM Na₂HPO₄, 50 mM HEPES, pH 7.0). Precipitates were formed by gradually adding 124 μ l of 1 M CaCl₂ solution and incubating the mixtures at room-temperature for 30 minutes.

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The suspension of precipitated DNA was gently added to two \varnothing 6 cm dishes containing each a semiconfluent monolayer of CRFK cells in 5 ml of culture medium. After 5 hours, medium was removed and 5 ml of HBSP with 15% glycerol was layered onto the cells. After a one to two minute incubation, the solution was removed, cells were washed with medium and dishes were incubated with overlayers of 0.75% agarose in culture medium. After 3 to 4 days when cytopathic effect started to develop, a second agarose overlay containing the substrate Bluogal (Gibco-BRL, Maryland, USA) with a final concentration of 0.2 mg/ml, was added and plates were incubated until blue plaques were detected. Positive plaques were picked macroscopically and transferred to flasks with fresh CRFK cells in order to amplify the virus. The plating procedure and plaque isolation was continued until homogeneous stocks of recombinant virus had been established. Virus material from the final preparations was used for detailed analysis of the viral genome by Southern blotting and for animal vaccination experiments.

Recombinant FHV containing the β -galactosidase marker gene inserted at the BglII site as present in pFHV13, was shown to be stable upon serial passage in tissue culture on CRFK cells.

A second site in the unique short segment of the FHV genome at which position the β -galactosidase gene could be inserted was mapped in the 5.2 kb BamHI restriction fragment of λ FHV04. A subclone containing this fragment in pGEM3Z and designated pFHV10, (see figure 3A), was partially digested with the restriction enzyme Sau3A, which has a four-base recognition sequence and generates cohesive DNA extremities compatible with the extremities generated by the enzymes BglII or BamHI.

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By including 10 $\mu\text{g/ml}$ of ethidium bromide in the Sau3A restriction digest, the digestion of linearized plasmid DNA into smaller fragments was inhibited.

Purification of the full size 7.9 kb linearized DNA of pFHV10 and ligation with the BamHI β -galactosidase expression cassette described above, generated recombinants containing the marker gene inserted randomly at one of the Sau3A restriction sites in pFHV10, including those containing the marker gene in one of the Sau3A sites of the 5.2 kb BamHI insert derived from λ FHV04.

One of the candidates selected from this experiment was shown to contain the marker gene inserted at the Sau3A site indicated in figure 3A.

This construct was designated pFHV23 and DNA of this plasmid was transfected with viral DNA into CRFK cells as described previously for pFHV19.

Recombinant FHV expressing β -galactosidase activity could be detected among the transfection progeny and these were purified to homogeneity following the procedures described above. Therefore, also the insertion of DNA at a position in the FHV genome corresponding to the Sau3A site indicated in figure 3A, does not interfere with functions essential for viral maintenance.

A detailed restriction map was derived from the complete sequence presented in SEQ ID NO: 1 and the exact position of the relevant open reading frames were indicated next to it. The resulting graph which is shown in figure 4, revealed several correctly positioned restriction sites both within ORF-2 and ORF-3. However, neither of these sites could be used directly for the insertion of a marker gene since the in Example 2 described β -galactosidase expression cassette is flanked by BamHI sites. The following modifications therefore had to be introduced first.

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Plasmid pFHV10 (see figure 1 and 3a) was chosen for all subsequent manipulations.

The first step consisted in removal of a 0.2 kb Bam HI-BglII fragment between mapposition 5100 and 5300 (see figure 3a) and recircularization of the remaining part with a size of ca. 7.7 kb, thereby suppressing both the BglII and BamHI site originally present in this part of the region. This deletion resulted in plasmid pFHV40 which contained only one BamHI site and none for BglII. Derivatives could now be made based on pFHV40 by insertion of synthetic double stranded linker molecules containing the BglII recognition sequence AGATCT at appropriate positions within the region defined by SEQ ID NO:1.

Two of these positions were selected based on one of the restriction sites either for EcoRV or Spe I, thereby allowing the insertion into ORF-2 and ORF-3, respectively as is shown in figure 5. Both enzymes cut at multiple positions in the sequence and the restriction digest therefore was done in the presence of ethidium bromide similar to the procedure described for Sau3A in Example 2, resulting in a majority of linearized full-size plasmid DNA molecules which could be purified by preparative agarose gel electrophoresis. DNA was recircularized by means of synthetic BglII-linkers which were blunt-ended for EcoRV or which contained CTAG-extremities in the case of SpeI digested DNA.

Insertion of the BglII site at the proper position in pFHV40 was verified by restriction analysis. Subsequently, the β -galactosidase expression cassette flanked by BamHI sites was inserted at the newly created BglII site following identical procedures as described in Example 2. Insertion of the marker gen into ORF-2 after creating a BglII site which replaced the EcoRV positioned at 2.1 kb (see figure 4), resulted in pFHV60 having a restriction map

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as shown in figure 5a. In the case of ORF-3, the marker gene was integrated by means of a BglII site newly created at the SpeI position around 3.1 kb and resulted in pFHV55 with the corresponding map shown in figure 5b.

DNA of both pFHV 60 and pFHV 55 was cotransfected with viral DNA into CRFK cells as described previously in Example 2. Recombinant FHV expressing β -galactosidase activity could be detected by Bluogal staining and virus was recovered by single plaque isolation using agarose overlayers. Recombinant viruses were passaged several times in cell culture and were shown to retain the β -galactosidase marker gene stably integrated both for the constructs derived from pFHV60 as well as from pFHV55

EXAMPLE 3

Structural analysis of the insertion region in the unique short segment of the FHV genome.

The nucleotide sequence analysis was performed on relevant parts of the 5.2 kb BamHI and 4.8 kb SacI restriction fragment shown in Fig. 1.

Fragments of λ FHV04 were subcloned in both orientations either in pGEM3Z or pSP72 (Promega, Wisconsin, USA).

Progressive deletions were introduced using the enzyme exonuclease III (Henikoff, S., Gene 28, 351-359, 1984) after double digestion of the plasmid DNA with the appropriate restriction enzymes creating a 5'- and 3'-overhanging extremity. The presence of a 3'-overhanging single strand extremity prevented the plasmid vector DNA from being degraded by exonuclease III. Samples of the reaction mixture were taken at 30 seconds intervals and treated according to Henikoff supra., generating recircularized DNA molecules which

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were transformed into competent E.coli cells. Plasmid DNA from minipreparations of individual colonies were analyzed by restriction mapping for the size of the deletion that was introduced in the original fragment. Series of candidates containing progressive deletions were analyzed by nucleotide sequencing on double stranded DNA in a chain termination reaction using T7 polymerase (Pharmacia, Uppsala, Sweden).

Incomplete or ambiguous readings within the nucleotide sequence were resolved by specific priming of the chain elongation reaction. Sequence data were assembled and analyzed using Gene-Master (Bio-Rad, California, USA) or equivalent software. Assemblage of all data resulted in an about 6.1 kb region (SEQ ID NO:1) within the unique short segment of the FHV genome consisting of six open reading frames encoding the respective polypeptides with amino acid sequences as shown in SEQ ID NO: 2, 3, 4, 5, 6 and 7.

The region of about 6.1 kb, consisting of the six open reading frames including interjacent and flanking, non-translated DNA sequences, can be applied for the insertion of foreign genes into the genome of FHV without disabling essential viral functions necessary for infection and replication.

Particularly the BglII restriction site at nucleotide position 1210 and the Sau3A site at 5737, which were used in Example 2 for the insertion of the β -galactosidase marker gene in pFHV13 and pFHV10, respectively, were shown to be mapping in positions of the FHV genome which are not essential for viral infection or replication.

EXAMPLE 4Pathogenicity of a FHV mutant in infected animals.

A recombinant FHV strain designated C4-1-4-1 and containing the β -galactosidase marker gene inserted at the BglIII site located within the COOH-terminal region of ORF-1 (see figure 1), was selected for evaluation in a cat vaccination trail and compared with the parent FHV strain G2620. The potential of C4-1-4-1 as a new FHV vaccine strain was assessed based on the pathogenecity and the ability of the virus to protect against the clinical signs caused by challenge infection with a virulent FHV strain.

Specific pathogen-free cats 12 weeks of age were infected oronasally with ca. 1×10^5 TCID₅₀ of the FHV mutant or parent strain by applying 0,3 ml per nostril and 0,4 ml in the oropharynx. Animals were observed daily over a period of 2 weeks for clinical signs speci-fic for FHV infection and scored based on the criteria as listed in Table 1. Cats were challenged six weeks after vaccination by oronasal application of 1×10^5 TCID₅₀ of FHV strain SGE (National Veterinary Service Laboratory, USA) and monitored over a period of 2 weeks for clinical signs of FHV infection.

Clinical observations both after vaccination and challenge are summarized in Table 2. Cats in group 1 that had received an oronasal vaccination with strain C4-1-4-1 showed a reduced level in the score for clinical signs compared to the animals in group 2 receiving the parent G2620 strain.

After challenge the vaccinated cats in group 1 still showed a strong reduction in the clinical scores compared to the non-vaccinated controls in group 3. Therefore it was concluded that the mutant strain C4-1-4-1, had a reduced virulence upon oronasal application in the cat and was still capable of inducing high levels of protection against the clinical signs of a challenge FHV infection.

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Table 1.

Clin. sign	Severity	Daily Score (points)
Pyrexia	>39.6 - 39.9	1
	40.0 - 40.4	2
	40.5 - 40.9	3
	>41.0	4
Sneezing	infrequent	1
	frequent	2
	paroxysmal	3
Cough	infrequent	1
	frequent	2
Respiration	URT noise	1
	stertor	1
	mouth breathing	2
Salivation		2
Conjunctivitis	mild	1 per eye
	moderate	2 per eye
	severe	3 per eye
Ocular discharge	serous	1 per eye
	mucopurulent	2 per eye
Nasal discharge	serous	1 per nostril
	mucopurulent	2 per nostril
Ulceration	nasal	2
	nasal/bleeding	3
	oral	2
	oral/bleeding	3
Oral erythema		1
Inappetance		1
Depression		

Table 2

group	vaccine	animal code	Clin. scores post-vaccination		Clin. scores post-challenge	
			individual score	average	individual scores	average
1	C4-1-4-1	40 O	6	1.7	14	12.0
		40 Q	0		9	
		40 Y	0		11	
		H 16	1		14	
2	G 2620	40 V	1	7.0	1	5.5
		40 W	8		7	
		40 X	5		6	
		H 18	14		8	
3	none	H 14	-	-	77	79.7
		H 12	-		85	
		H 11	-		69	
		B 39	-		88	

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EXAMPLE 5Construction of recombination plasmid for the insertion of heterologous genes into the genome of FHV.

The recombination plasmid used for the insertion and subsequent expression of foreign genes in FHV was based on the BglII restriction site at nucleotide position 1210 (SEQ ID NO: 1) and mapped in ORF-1 (figure 1). The 5.2 kb BamHI fragment from λ FHV04 was thereto subcloned in the BglII site of pSP72 (Promega, Wisconsin, USA) such that the orientation of ORF-1 was identical to the orientation of the T7 RNA polymerase promotor of the plasmid vector. This construct, designated pFHV11, was submitted to the uni-directional deletion technique using the enzyme exonuclease III and following similar procedures as described in example 3 for the nucleotide sequence analysis. This resulted in pFHV27 which contains a remaining 0,4 kb from the original 5.2 kb BamHI fragment with the unique BglII site positioned about in the middle and with sufficient flanking FHV genomic sequences in order to allow the in vivo recombination with the viral genome.

A strong promotor which could direct the expression of foreign genes after their insertion into the genome of the FHV virus was selected from the LTR sequence of Rous sarcoma virus (RSV). The promotor has been mapped on a 580 bp NdeI/HindIII restriction fragment from pRSVcat (Gorman, C. M. et al., Proc. Natl. Acad. Sci. USA 79, 6777-6781, 1982) and was inserted between the HindIII and PstI sites of pGEM3Z by means of double stranded synthetic linkers on both sides of the fragment. The connection between the HindIII site from the vector pGEM3Z and the NdeI site of the RSV fragment carrying the LTR promotor was made with a 30 bp linker containing cohesive ends compatible with HindIII on one and NdeI on the other site. However, after ligation both restriction

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sites are not restored due to deliberate modifications in the outer nucleotides of the six basepair recognition sequence. In addition to the removal of these two sites, a new restriction site (BamHI) present within the linker itself was created at the corresponding position. A second 20 bp linker was synthesized which connected the HindIII site from the LTR fragment to the PstI site from pGEM3Z, in this case without destruction of the recognition sequence on either of the ends and adding the convenient restriction sites BglII and XhoI, to those already present in the polylinker of pGEM3Z. The resulting derivative, designated pVEC01, therefore contains a 650 bp restriction fragment carrying the LTR promotor sequence immediately followed by multiple restriction sites available for the insertion of foreign genes. The 650 bp fragment is flanked on either end by a BamHI restriction site and has been transferred as such to the unique BglII site present in pFHV27. The cohesive ends generated by these two restriction enzymes are compatible but ligation does not restore either of the original recognition sequences for BglII or BamHI. The resulting constructs was designated pFHV38 and checked by restriction mapping (figure 6). The structure of this FHV recombination vector allows the insertion of foreign genes immediately downstream of the LTR promotor and subsequent integration of the complete expression cassette into the FHV genome by in vivo recombination. The positions of the different restriction sites downstream of the LTR in particular those for the enzymes BglII and SalI, are designed in such a way that even multiple gene insertion can be envisaged.

LEGENDSFigure 1

Restriction map of the 13.5 kb DNA insert from λ FHV04. The position of this DNA fragment is mapped in the right-ward part of the unique short region of the FHV genome. The insertion region is composed of the six open reading frames indicated at the top including intergenic non-translated sequences. The subcloning of the 4.8 kb SacI and 5.2 kb BamHI restriction fragments in pGEM3Z resulted in pFHV13 and pFHV10, respectively. The sequence analysis indicated that the most left-ward EcoRI restriction site contained two six-base recognition sequences which were located within 50 bp from each other.

Figure 2

- A Restriction map of plasmid pFHV13, a derivative of pGEM3Z containing the 4.8 kb SacI from λ FHV04. The unique BglII at 3.1 kb, which was used for inserting DNA, is labelled with a triangle.
- B Restriction map of plasmid pFHV19, derived from pFHV13 by insertion of a 4.0 kb BamHI fragment containing the β -galactosidase marker gene.

Figure 3

- A Restriction map of plasmid pFHV10, a derivative of pGEM3Z containing the 5.2 kb BamHI fragment from λ FHV04. The Sau3A site labelled with a triangle was used for the insertion of DNA.
- B Restriction map of plasmid pFHV23, derived from pFHV10 by insertion of a 4.0 kb BamHI fragment containing the β -galactosidase marker gene.

Figure 4

Detailed restriction map derived from the sequence listed in SEQ ID NO:1. Positions of the six

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open reading frames are indicated in the top. The EcoRV site at 2.1 kb was used for insertion of the marker gene into ORF-2 by contranfections of viral DNA with the plasmid pFHV60.

The Spe I site at 3.1 kw was used in a similar manner to insert the marker gene into ORF-3. In this case, plasmid pFHV55 was used in the cotransfections.

Figure 5

Restriction map of pFHV60 (A) and pFHV55 (B) which were both derived from pFHV40. This plasmid was derived from pFHV10 by deletion of a 0.2 kb Bam HI-BgIII fragment around 5.1 kb (figure 3A), such that both restriction sites were not restored after ligation.

A. Restriction map of pFHV60, a derivative of pFHV40 with an insertion of the β -galactosidase gene at a position equivalent to the EcoRV site which is indicated in figure 4. This insertion disrupts the coding sequence of ORF-2.

B. Restriction map of pFHV55, derived from pFHV40 by insertion at the Spe I site shown in figure 4.

The coding sequence of ORF-3 is disrupted in this construct.

Figure 6

Restriction map of pFHV38. The in vivo recombination vector contains the LTR promotor necessary for the expression of foreign genes which can be inserted downstream of the promotor at the restriction site e.g. BglII or SalI.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: AKZO NV
- (B) STREET: Velperweg 76
- (C) CITY: Arnhem
- (E) COUNTRY: the Netherlands
- (F) POSTAL CODE (ZIP): 6824 BM

(ii) TITLE OF INVENTION: Vector vaccines of recombinant
Feline herpesvirus

(iii) NUMBER OF SEQUENCES: 7

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version
#1.25 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6154 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Feline herpesvirus (FHV-1)
- (B) STRAIN: G2620

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 127..1281
- (D) OTHER INFORMATION: /label= ORF-1

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1460..3058
- (D) OTHER INFORMATION: /label= ORF-2

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 3055..3357
- (D) OTHER INFORMATION: /label= ORF-3

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(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 3505..3963
- (D) OTHER INFORMATION: /label= ORF-4

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: complement (4256..4897)
- (D) OTHER INFORMATION: /label= ORF-5

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: complement (5138..6142)
- (D) OTHER INFORMATION: /label= ORF-6

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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TATCCAACAC TGGAGAATTT TACGATCTAC GGCCATCTAA TCCTTCTCGA CGACCAACCA	300
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TATTCAGGGG GCCACTACGA ATATATCCGG GATATATACC CTGCATGAGC ACGGTGATAA 1920
TGGATGGAGT CATCAATCTA CATTTTTTGT GACCGTAAAG GCAAAACATC CCGGACCATC 1980

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SUBSTITUTED SEQUENCE

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 384 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) FEATURE:

(D) OTHER INFORMATION: /label=ORF-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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 1               5               10               15
Tyr Gly Ile Val Tyr Arg Gly Asp His Val Ser Leu His Val Asp Thr
      20               25               30
Ser Ser Gly Phe Val Ile Tyr Pro Thr Leu Glu Asn Phe Thr Ile Tyr
      35               40               45
Gly His Leu Ile Phe Leu Asp Asp Gln Pro Leu Pro Val Asn Asn Tyr
      50               55               60
Asn Gly Thr Leu Glu Ile Ile His Tyr Asn His His Ser Ser Cys Tyr
      65               70               75               80
Lys Ile Val Gln Val Ile Glu Tyr Ser Ser Cys Pro Arg Val Arg Asn
      85               90               95
Asn Ala Phe Arg Ser Cys Leu His Lys Thr Ser Met His Gln Tyr Asp
      100               105               110
Gln Leu Ser Ile Asn Thr Ser Val Glu Thr Gly Met Leu Leu Thr Ile
      115               120               125
Thr Ser Pro Lys Met Glu Asp Gly Gly Ile Tyr Ala Leu Arg Val Arg
      130               135               140
Phe Asn His Asn Asn Lys Ala Asp Val Phe Gly Leu Ser Val Phe Val
      145               150               155               160
Tyr Ser Phe Asp Thr Arg Gly His Arg His His Ala Asp Glu Asn Leu
      165               170               175

```

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Asn	Gly	Glu	Ile	Leu	Thr	Thr	Pro	Ser	Ser	Met	Glu	Thr	Tyr	Val	Lys
			180					185					190		
Val	Asn	Thr	Pro	Ile	Tyr	Asp	His	Met	Val	Thr	Thr	Gln	Thr	Thr	Ser
		195					200					205			
Asn	Lys	Ser	Met	Glu	Ser	Glu	Pro	Ser	Asn	Thr	Ser	Ile	Ser	Cys	His
	210					215					220				
Thr	Phe	Gln	Asn	Asp	Pro	Asn	Glu	Gly	Glu	Thr	Leu	Tyr	Thr	His	Leu
225					230					235					240
Leu	Asn	Ile	Ala	Gly	Asn	Ile	Thr	Tyr	Asp	Asp	Met	Val	Met	Asp	Gly
				245					250					255	
Thr	Thr	Leu	Gln	Pro	Arg	Leu	Ile	Asp	Met	Gly	Leu	Asn	Leu	Ser	Val
			260					265					270		
Thr	Ser	Ser	Phe	Lys	Asn	Glu	Thr	Thr	Gln	Lys	Trp	Thr	Pro	Asp	Arg
		275					280					285			
Lys	Val	Gly	Phe	Val	Ile	Val	Ile	Ser	Ile	Ala	Val	Leu	Leu	Leu	Leu
	290					295					300				
Ala	Val	Ile	Gly	Ser	Ile	Ile	Asn	Ser	Ala	Ile	Arg	Lys	His	Ile	Met
305					310					315					320
Val	Cys	Ala	Gly	Arg	Arg	Ile	Tyr	Ile	Pro	Asn	Asn	Asp	Gly	Arg	Pro
				325					330					335	
Ser	Thr	Glu	Met	Thr	Arg	Phe	Thr	Arg	Gln	Thr	Lys	Pro	Ser	Asn	Ser
			340					345					350		
Ser	Ser	Lys	Ser	Leu	Leu	Asp	Val	Pro	Arg	Ser	Ser	Asn	Ser	Thr	Pro
		355					360					365			
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	370					375					380				

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 532 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) FEATURE:

- (D) OTHER INFORMATION: /label=ORF-2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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Met	Gly	Leu	Leu	Val	Thr	Ile	Leu	Val	Ile	Leu	Leu	Ile	Val	Thr	Ser	1	5	10	15
Ser	Ser	Ser	Thr	Ile	His	Gln	Val	Thr	Met	Thr	Glu	Gly	Ala	Ala	Leu	20	25	30	
Leu	Val	Asp	Gly	Asp	Gly	Ile	Asp	Pro	Pro	Leu	Asn	Lys	Thr	Ser	His	35	40	45	
Phe	Leu	Arg	Gly	Trp	Thr	Phe	Leu	Glu	Thr	Pro	Lys	Gly	Cys	Thr	Gly	50	55	60	
Glu	Val	Ser	Val	Leu	Lys	Val	Cys	Ile	Asp	Arg	Gly	Val	Cys	Pro	Asp	65	70	75	80
Asp	Ile	Val	Ile	Asn	Lys	Arg	Cys	Gly	His	Lys	Met	Leu	Glu	Thr	Pro	85	90	95	
Leu	Ala	Leu	Ala	Glu	Leu	Gly	Ile	Ser	Asn	Ser	Ser	Leu	Ile	Arg	Thr	100	105	110	
Lys	Asp	Val	Tyr	Phe	Val	Asn	Lys	Thr	Val	Phe	Pro	Ile	Leu	Thr	Pro	115	120	125	
Glu	Lys	Ser	Gly	Leu	Gly	Ile	Gln	Gly	Ala	Thr	Thr	Asn	Ile	Ser	Gly	130	135	140	
Ile	Tyr	Thr	Leu	His	Glu	His	Gly	Asp	Asn	Gly	Trp	Ser	His	Gln	Ser	145	150	155	160
Thr	Phe	Phe	Val	Thr	Val	Lys	Ala	Lys	His	Pro	Gly	Pro	Ser	Leu	Thr	165	170	175	
Pro	Ala	Pro	Val	His	Leu	Ile	Thr	Pro	His	Arg	His	Gly	Ala	His	Phe	180	185	190	
His	Val	Arg	Asn	Tyr	His	Ser	His	Val	Tyr	Ile	Pro	Gly	Asp	Lys	Phe	195	200	205	
Leu	Leu	Glu	Met	His	Leu	Lys	Ser	Asp	Ile	Tyr	Asp	Pro	Glu	Phe	Ser	210	215	220	
Ala	Thr	Ile	Asp	Trp	Tyr	Phe	Met	Glu	Thr	Asp	Ile	Lys	Cys	Pro	Val	225	230	235	240
Phe	Arg	Ile	Tyr	Glu	Thr	Cys	Ile	Phe	His	Pro	His	Ala	Ala	Ser	Cys	245	250	255	
Leu	His	Pro	Glu	Asp	Pro	Ser	Cys	Ser	Phe	Thr	Ser	Pro	Leu	Arg	Ala	260	265	270	
Val	Ser	Leu	Ile	Asn	Arg	Phe	Tyr	Pro	Lys	Cys	Asp	His	Arg	Tyr	Ala	275	280	285	
Asp	Trp	Thr	Ser	Arg	Cys	Ile	Asn	Thr	Pro	Ser	Ile	Asn	His	Met	Pro	290	295	300	

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Tyr Ile Glu Gln Pro Ala Asn Asn Val Asp Leu Lys Phe Ile Asn Val
 305 310 315 320
 Pro Thr Asn Ala Ser Gly Leu Tyr Val Phe Ile Leu Arg Tyr Asn Gly
 325 330 335
 His Pro Glu Glu Trp Thr Tyr Thr Leu Thr Ser Thr Gly Ala Lys Phe
 340 345 350
 Leu Asn Val Ile Arg Asp Leu Thr Arg Pro Arg Leu Gly Ser His Gln
 355 360 365
 Ile Glu Thr Asp Ile Ser Thr Ser Ser Gln Ser Pro Thr Thr Glu Thr
 370 375 380
 Pro Arg Asn Ile His Ile Thr Trp Ala Arg Arg Tyr Leu Lys Val Ile
 385 390 395 400
 Ile Gly Ile Ile Cys Val Ala Gly Ile Leu Leu Ile Val Ile Ser Ile
 405 410 415
 Thr Cys Tyr Ile Arg Phe Arg His Met Arg Tyr Lys Pro Tyr Glu Val
 420 425 430
 Ile Asn Pro Phe Pro Ala Val Tyr Thr Ser Ile Pro Ser Asn Asp Pro
 435 440 445
 Asp Glu Leu Tyr Phe Glu Arg Ile Ala Ser Asn Asp Glu Glu Ser Ala
 450 455 460
 Asp Asp Ser Phe Asp Glu Ser Asp Glu Glu Glu Pro Leu Asn Asn His
 465 470 475 480
 His Ile Ser Thr Thr Gln His Thr Asp Ile Asn Pro Glu Lys Ser Gly
 485 490 495
 Ser Gly Tyr Ser Val Trp Phe Arg Asp Thr Glu Asp Thr Ser Pro Gln
 500 505 510
 Pro Leu His Ala Pro Pro Asp Tyr Ser Arg Val Val Lys Arg Leu Lys
 515 520 525
 Ser Ile Leu Lys
 530

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 100 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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(ix) FEATURE:

(D) OTHER INFORMATION: /label=ORF-3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```

Met Thr Arg Arg Arg Val Leu Ala Pro Arg Glu Leu Glu Ala Ala Arg
 1           5           10           15
Lys Leu Arg Glu Ile Phe Asn Ala Glu Tyr Val Ala Pro Thr Phe Thr
          20           25           30
Leu Val Asp Pro Gly Asp Thr Ser Asn Ala Tyr Ile Val Cys Arg Thr
          35           40           45
Pro Val Thr Glu Val Val Ser Ser Ile Ser Arg Gly Ile Asp Asn Arg
          50           55           60
Lys Ser Val Asp Ser Ser Phe Ile Arg Ile Val Ser Lys Leu Ile Ile
 65           70           75           80
Arg Asn Ala Ile His Met Gly Leu Ser Val Leu Cys Ala Phe Ile Ser
          85           90           95
Tyr Asn Lys Pro
          100

```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 152 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) FEATURE:

(D) OTHER INFORMATION: /label=ORF-4

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```

Met Asp His Gln Thr Ser Leu Ile Asn Ala Thr Asp Asp Asn Cys Leu
 1           5           10           15
Asp Thr Asp Ser Ser Ile Asn Leu Pro Ser Ile Asp Lys Cys Glu Ile
          20           25           30
Asp Asp Asn Ser Ile Ala Asp Glu Thr Leu Ser Asp Lys Gly Ser Pro
          35           40           45
Val Ala Ile Pro Leu Cys Ala Thr Ile Glu Ile Pro Arg Gly Asn Ala
          50           55           60
Asp Arg Gln Ser Pro Ser His Asp Val Arg Gly Ala Asn Arg Thr Asn
 65           70           75           80

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Tyr	Asp	Ser	Asp	Thr 85	Gly	Cys	Tyr	Tyr	Ser 90	Glu	Ser	Asp	Asn	Glu 95	Thr
Ala	Thr	Leu	Phe 100	Ile	Asn	Arg	Ile	Gly 105	Lys	Arg	Glu	Thr	Ala 110	Lys	Arg
Arg	Arg	Arg 115	Arg	Arg	Cys	Leu	Val 120	Ala	Leu	Ala	Val	Ser 125	Gly	Val	Ala
Thr	Leu 130	Cys	Val	Leu	Ser	Gly 135	Leu	Leu	Gly	Ala	Leu 140	Leu	Trp	Arg	Leu
Met 145	Asp	Ala	Pro	Gly	Thr 150	Arg	Arg								

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 213 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met 1	Asn	Arg	Arg	Trp 5	Glu	Asp	Thr	Asn	Ile 10	Glu	Ser	Phe	Asn	Met 15	Thr
Gly	Val	Ala	Glu 20	Met	Glu	Met	Tyr	Pro 25	Leu	Arg	Gly	Asp	Ser 30	Ala	Asp
His	Ala	Glu 35	Thr	Leu	Pro	Arg	Ser 40	Val	Arg	Ala	Leu	Phe 45	Asp	Ala	Leu
Arg	Val 50	Ala	Ser	Cys	Glu	Ala 55	Phe	Cys	Leu	Met	Arg 60	Leu	Gly	Gly	Pro
Pro 65	Pro	Ala	Asp	Ile	Trp 70	Pro	Gly	Val	Tyr	Arg 75	Gln	Tyr	Arg	Glu	Val 80
Phe	Arg	Ser	Tyr	Ser 85	Arg	Ser	Met	Glu	Gly 90	Ser	Gly	Gly	Ser	Pro 95	Phe
His	Val	Ala	Asp 100	Pro	Ile	Arg	His	Leu 105	Val	Gly	Arg	Tyr	Leu 110	Met	Gly
Leu	Gly	Pro 115	Ala	Lys	Pro	Glu	Ser 120	His	Pro	Glu	Leu	His 125	Thr	Arg	Leu

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Leu Tyr Cys Ala Tyr Trp Cys Cys Leu Gly His Ala Ala Thr Cys Thr
 130 135 140
 His Ser His Ile Tyr Glu Asp Ala Cys Arg Arg Phe Phe Glu Glu Gly
 145 150 155 160
 Phe Gly Ala Gly Glu Ile Pro Pro Ala Asp Ala Val Ala His Trp Asn
 165 170 175
 Ala Leu Tyr Glu Met Val Leu Asp Glu Pro Glu Leu Leu Val Lys His
 180 185 190
 Ala Ala Ala Ala Val Tyr Leu Gln Arg Arg Asn Tyr Gly Gly Cys Ile
 195 200 205
 Pro Asn Ile Glu Lys
 210

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 334 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) FEATURE:

- (D) OTHER INFORMATION: /label=ORF-6

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Met Ala Lys Gly Arg Lys His Arg Gly Arg Pro His Thr Thr Pro Ala
 1 5 10 15
 Ala Val Lys Pro Pro Glu Leu Arg Ser Gly Arg Leu Gly His Gly Gln
 20 25 30
 Pro Cys Gly Leu Cys Asp Gly Ser Cys Arg Leu Arg Pro Arg Gly Gly
 35 40 45
 Leu Asn Ile Asp Pro Ser Pro Ile Leu Pro Ser Leu Thr Pro Ser Thr
 50 55 60
 Gly Ser Ser Thr Phe Ala Ser Gln Thr Pro Gly Gly Ser Lys Ile Pro
 65 70 75 80
 Val Ser Cys Val Glu Thr Asp Asp Thr Ser Pro Val Arg Ala Pro Ala
 85 90 95
 Thr Leu Pro Asp Gly Asp Asp His Pro Glu Tyr Gly Ser Val Val Ala
 100 105 110

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Ser	Arg	Asp	Leu	Arg	Ala	Tyr	Leu	Thr	His	Gly	Pro	Gly	Ala	Phe	Cys
		115					120					125			
Ser	Pro	Pro	Trp	Arg	Cys	Asp	Leu	Lys	Arg	Leu	Met	Lys	Asp	Val	Asn
	130					135					140				
Ile	Met	Phe	Arg	Cys	Ile	Ala	Thr	Ser	Ser	Val	Asn	Val	Ser	Ser	Asp
145					150					155					160
Ser	Arg	Ala	Leu	Arg	Arg	Ala	Leu	Leu	Asp	Phe	Tyr	Ile	Met	Gly	Tyr
				165					170					175	
Thr	Asn	Gln	Arg	Pro	Thr	Arg	Ala	Cys	Trp	Glu	Thr	Leu	Leu	Gln	Leu
			180					185					190		
Ser	Ser	Glu	Gln	Ala	Arg	Pro	Leu	Arg	Ala	Thr	Leu	Arg	His	Leu	Asn
		195					200					205			
Thr	Thr	Glu	Pro	Cys	Asp	Arg	Arg	Phe	Leu	Glu	Pro	Pro	Thr	Gln	Val
	210					215					220				
Pro	Pro	Ile	Leu	Phe	Gly	Gln	Glu	Cys	Asp	Val	Ser	Gly	Ser	Glu	Asp
225					230					235					240
Ser	Glu	Asp	Gly	Glu	Gly	Val	Asp	Ile	Glu	Ser	Ser	Asp	Ser	Asp	Cys
				245					250					255	
Gly	Glu	Thr	Ser	Leu	Ser	Glu	Phe	Ser	Tyr	Val	Gly	Gly	Asp	Gly	Asp
			260					265					270		
Glu	Thr	Ser	Thr	Ser	Asp	Ser	Asp	Ser	Gly	Thr	Asp	Asp	Asp	Ser	Asp
		275					280					285			
Glu	Thr	Glu	Asp	Arg	Ser	Ser	Ser	Ser	Ser	Glu	Ser	Asp	Ser	Ser	Asp
	290					295					300				
Val	Asp	Tyr	Gly	Thr	Arg	Val	Cys	Gly	Lys	Lys	Arg	Arg	Cys	Asn	Pro
305					310					315					320
Val	Arg	Arg	Ser	Ala	Arg	His	Ala	Ala	Lys	Arg	Lys	Arg	Met		
				325					330						

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Claims

1. FHV mutant comprising a mutation in a section of an FHV genome which spans from the upstream non-coding region of open reading frame-1 up to and including the downstream non-coding region of open reading frame-6 localized within a DNA fragment of the FHV genome having a restriction enzyme map essentially defined by figure 1.
2. FHV mutant according to claim 1, characterized in that said section spans the region comprising the DNA sequences of the consecutive open reading frames ORF-1 to ORF-6 encoding the polypeptides shown in SEQ ID NO: 2-7 or variants thereof.
3. FHV mutant according to claim 1 or 2, characterized in that said section has the DNA sequence shown in SEQ ID NO: 1.
4. FHV mutant according to claims 1-3, characterized in that the mutation comprises an insertion of a heterologous DNA sequence.
5. FHV mutant according to claim 4, characterized in that the heterologous DNA sequence encodes a polypeptide and is under control of a promotor regulating the expression of said DNA sequence in a cell infected with said FHV mutant.
6. FHV mutant according to claims 4 or 5, characterized in that the heterologous DNA sequence encodes an antigen of a feline pathogen.

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7. FHV mutant according to claim 6, characterized in that the antigen is derived from a pathogen selected from the group consisting of feline leukemia virus, feline immunodeficiency virus, feline calicivirus, feline parvovirus, feline coronavirus and feline Chlamydia.
8. FHV mutant according to claims 1-7, characterized in that at least part of the DNA sequence of said section of the FHV genome is deleted.
9. Nucleic acid sequence comprising at least part of the section of an FHV genome defined in claim 1.
10. Nucleic acid sequence comprising a heterologous DNA sequence flanked by DNA sequences derived from a section of an FHV genome defined in claim 1.
11. Recombinant DNA molecule comprising a nucleic acid sequence according to claim 9 or 10.
12. Host cell transfected with a recombinant DNA molecule according to claim 11.
13. Cell culture infected with an FHV mutant according to claims 1-8.
14. Vaccine comprising an FHV mutant according to claims 1-8.
15. Method for the immunization of animals against an infectious disease which comprises administering a vaccine according to claim 14.

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FIG. 1

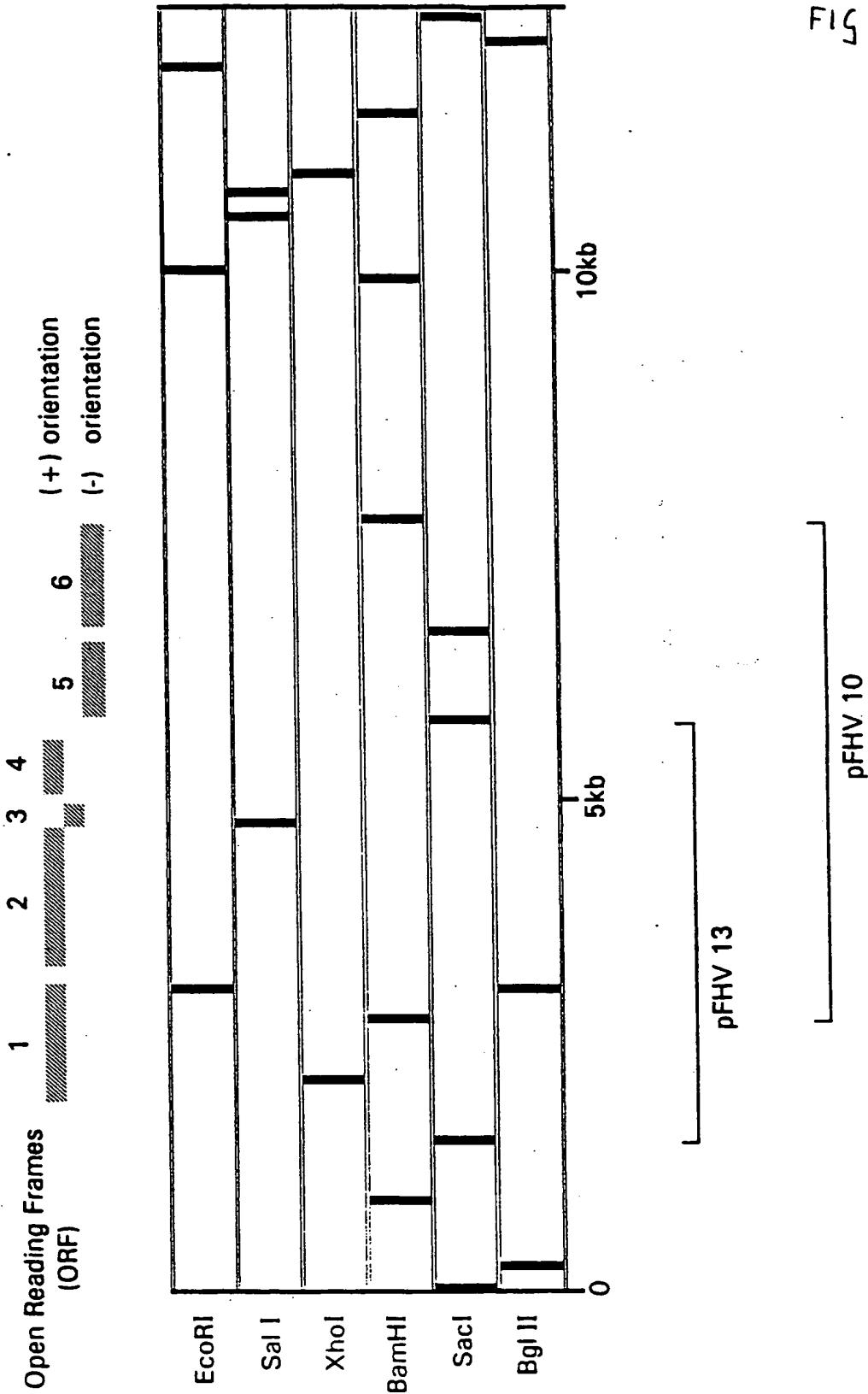
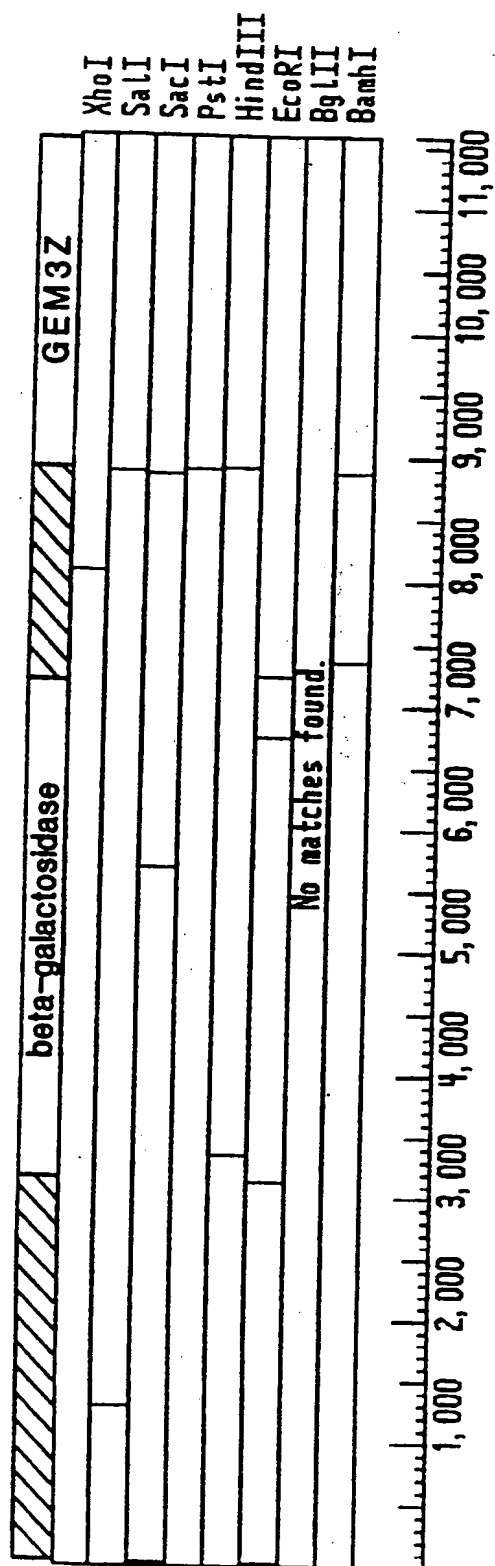
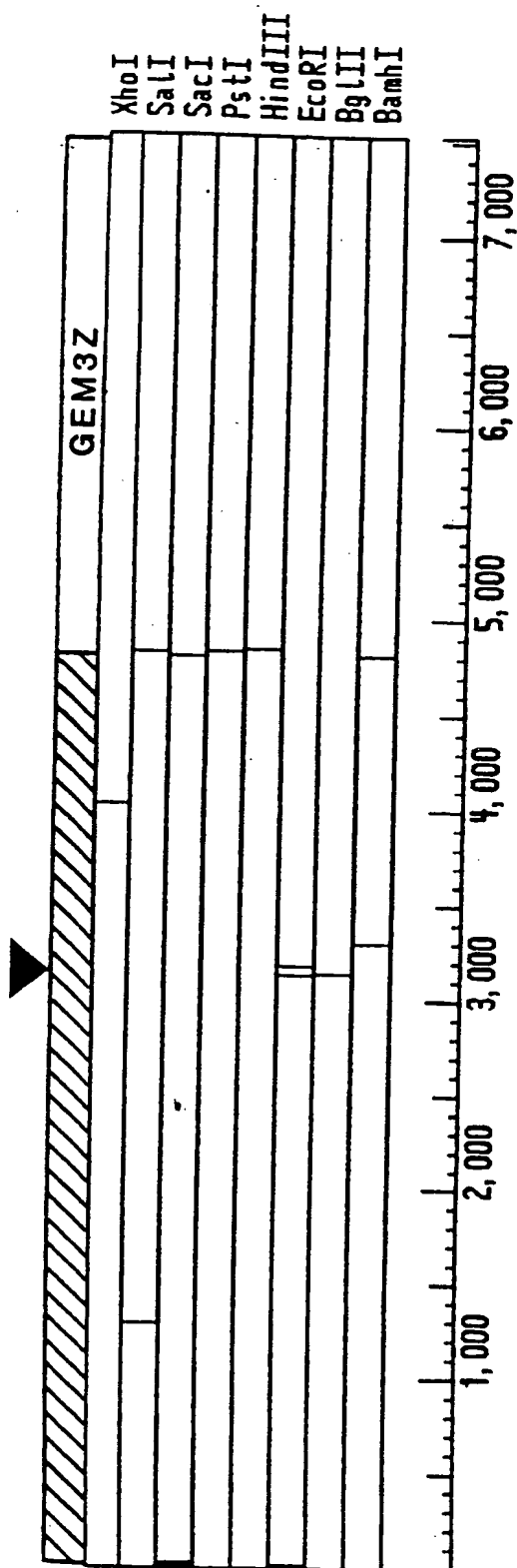
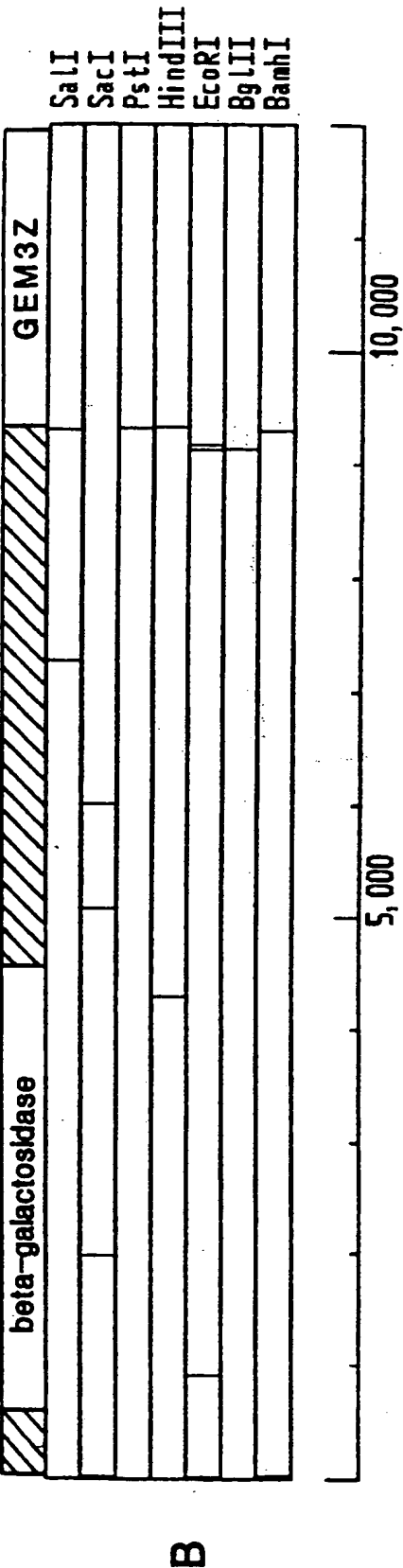
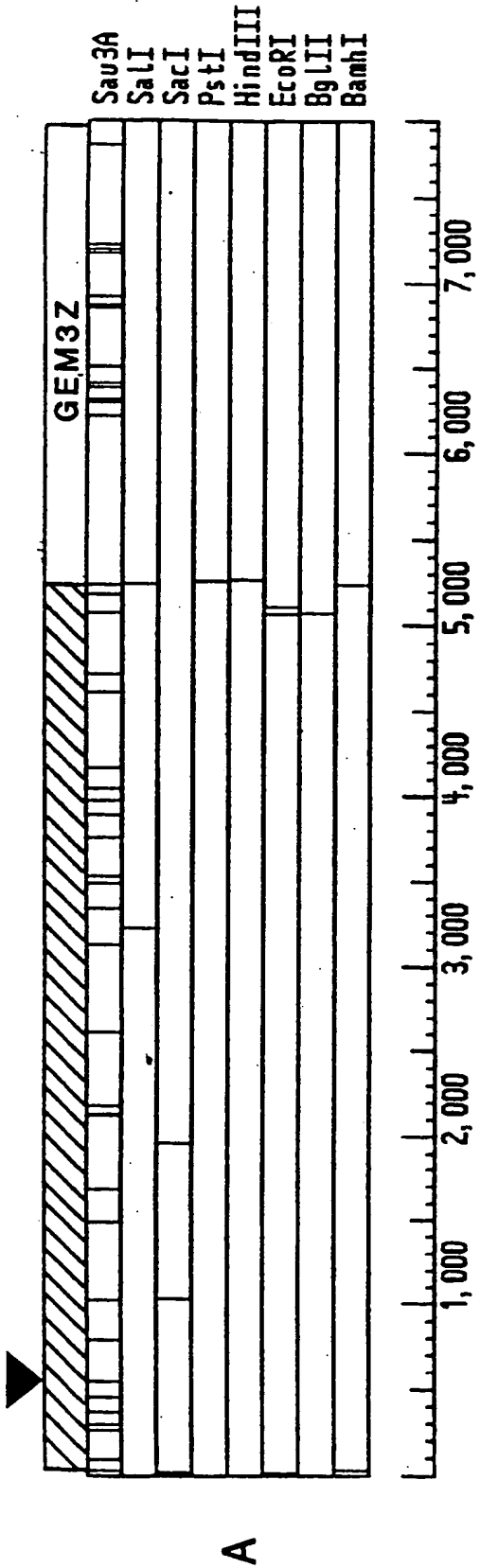


FIG. 2



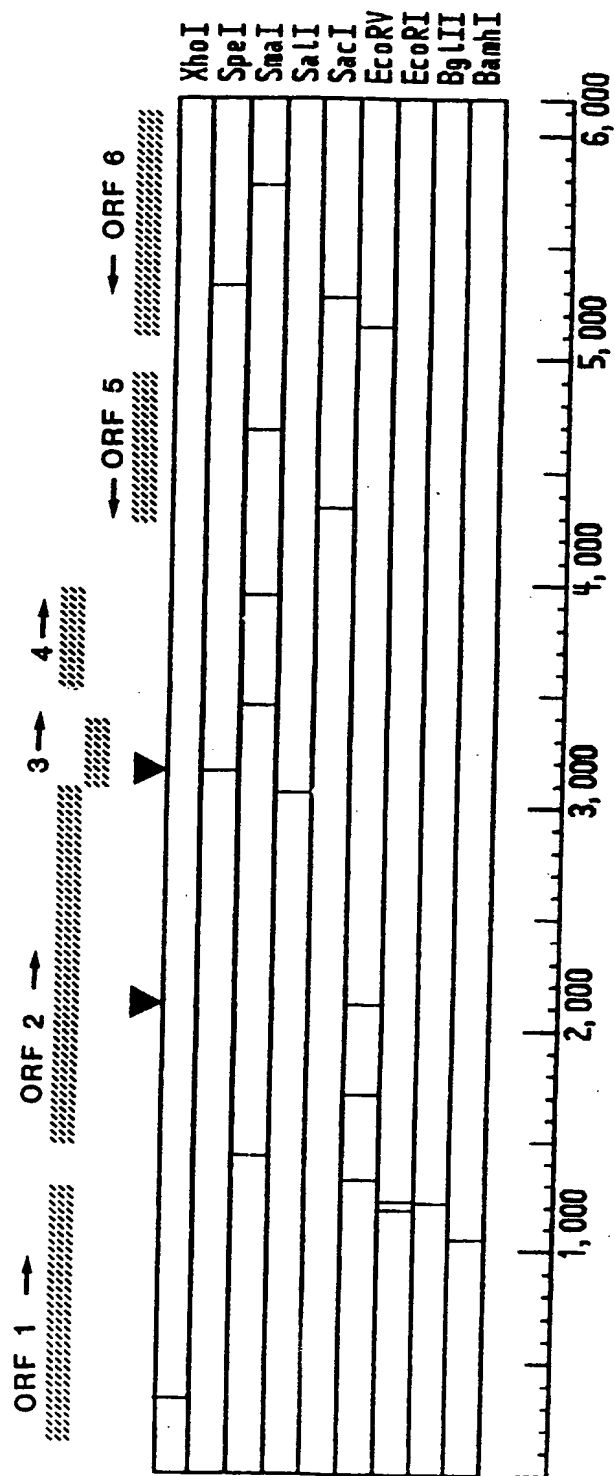
////// FHV homologous region

Fig. 3



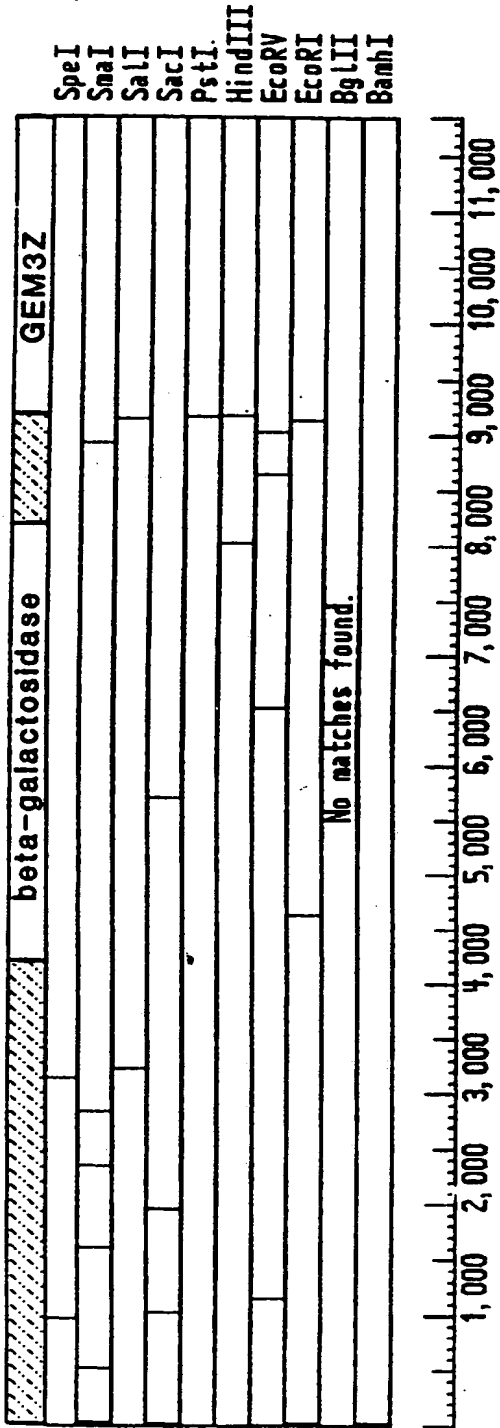
////// FHV homologous region

FIG. 4

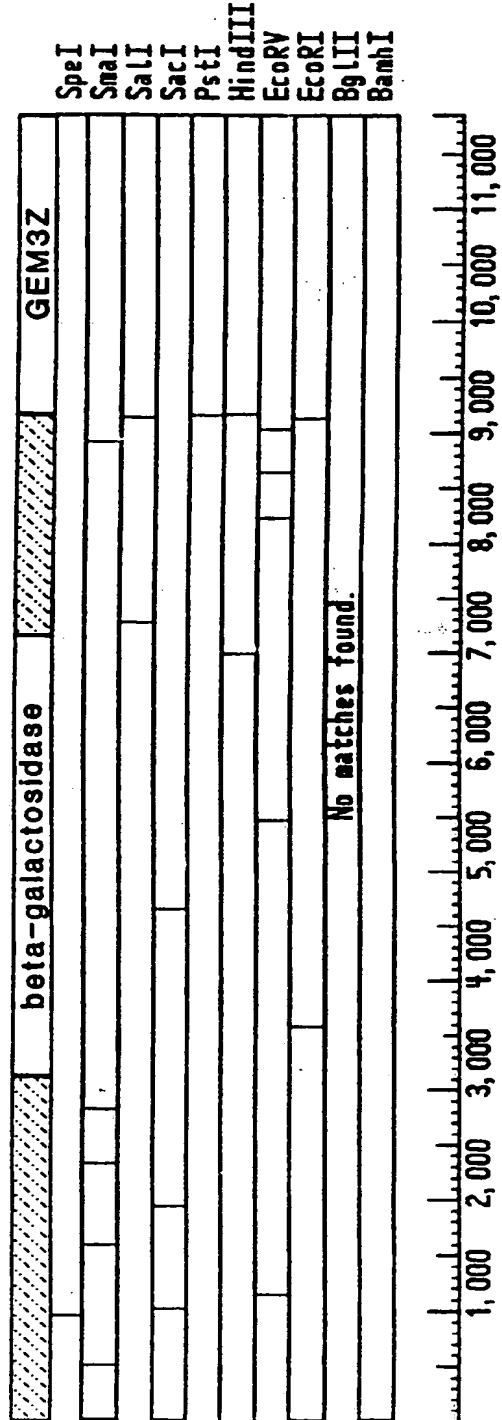


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Fig. 5



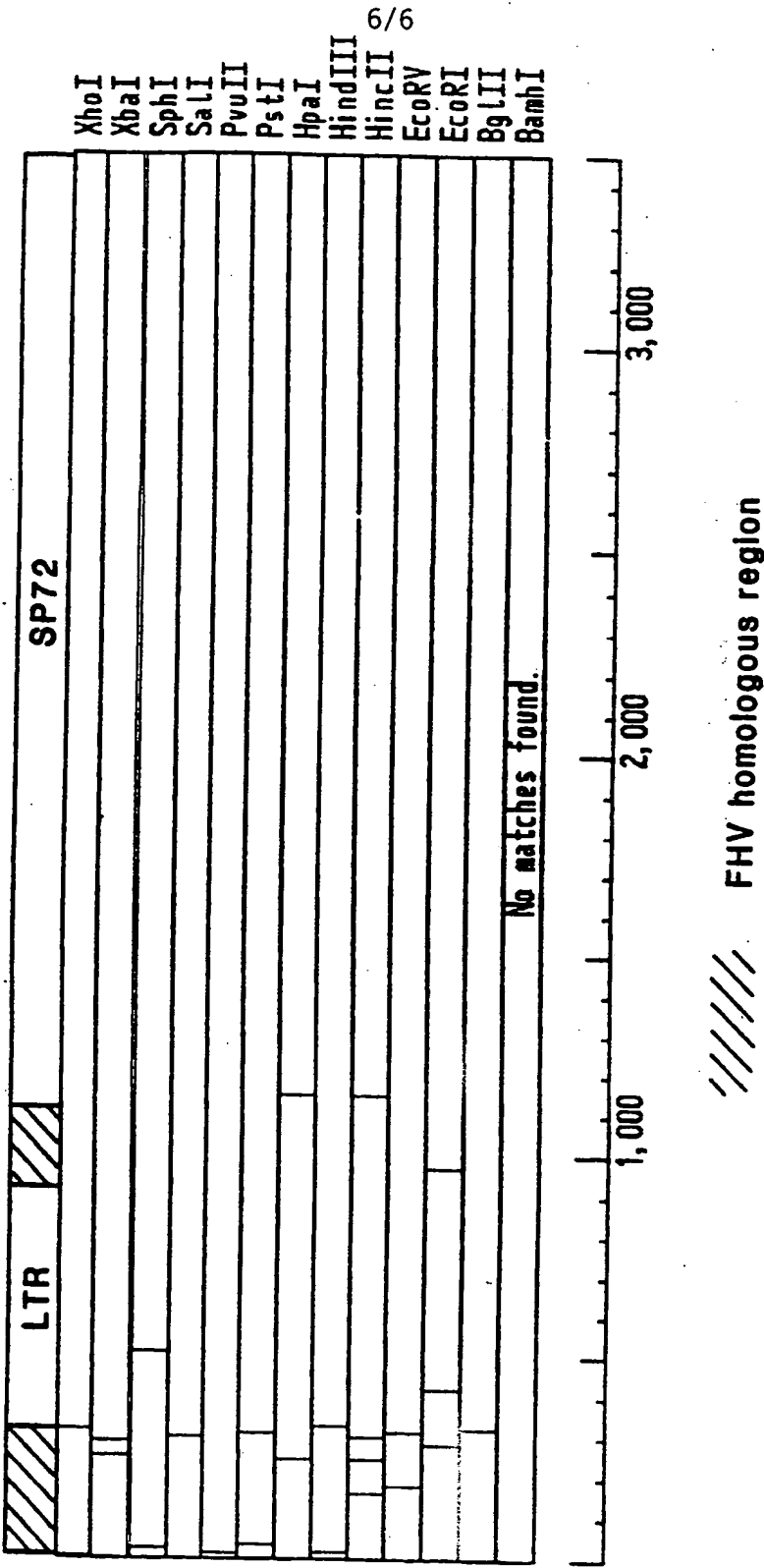
(A)



(B)

FHV homologous region

FIG. 6



INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 93/01971

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC Int.C1.5 C 12 N 15/86 C 12 N 15/38		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.C1.5	C 12 N C 07 K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y	EP,A,0431668 (AKZO N.V.) 12 June 1991, the whole document, in particular page 2, line 54 - page 3, line 22, page 5, example 1, page 8, example 3 ---	1-15
Y	JOURNAL OF GENERAL VIROLOGY, vol. 71, 1990, pages 2969-2978, Reading, Berks, GB, J.C. AUDONNET et al.: "Equine herpesvirus type 1 unique short fragment encodes glycoproteins with homology to herpes simplex virus type 1 gD, gI and gE", the whole document, in particular page 2977, column 1 --- -/-	1-15
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
04-10-1993		15 -11- 1993
International Searching Authority		Signature of Authorized Officer
EUROPEAN PATENT OFFICE		A. DE KOK

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	ARCHIVES OF VIROLOGY, vol. 116, nos. 1-4, 1991, pages 209-220, Wien, AT, A. GRAIL et al.: "Restriction endonuclease mapping of the genome of feline herpesvirus type 1", the whole document (cited in the description) ---	1-3,9
A	VIROLOGY, vol. 154, no. 1, 15 October 1986, pages 168-179, New York, US, P.A. ROTA et al.: "Physical characterization of the genome of feline herpesvirus-1", the whole document (cited in the description) ---	1-3,9
A	WO,A,8704463 (SYNTRO CORPORATION) 30 July 1987, the whole document ---	1-15
A	EP,A,0477056 (RHONE MERIEUX) 18 September 1991, the whole document -----	1-15

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

EP 9301971

SA 77266

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 14/10/93. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0431668	12-06-91	AU-B- 633663	04-02-93
		AU-A- 6769890	06-06-91
		CN-A- 1052896	10-07-91
		JP-A- 5103667	27-04-93
		US-A- 5187087	16-02-93
WO-A- 8704463	30-07-87	US-A- 5068192	26-11-91
		AU-A- 1026692	14-05-92
		AU-B- 623333	14-05-92
		AU-A- 7026687	14-08-87
		EP-A- 0256092	24-02-88
		JP-T- 63502482	22-09-88
		US-A- 5047237	10-09-91
		US-A- 5223424	29-06-93
EP-A- 0477056	25-03-92	FR-A, B 2601689	22-01-88
		FR-A- 2666589	13-03-92
		AU-A- 8370891	12-03-92
		CA-A- 2050850	08-03-92
		JP-A- 5168473	02-07-93

EP 9301971

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

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